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Citrullination – small change with a great consequence

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ABSTRACT

Citrullination is one of the possible post-translational modifications of proteins. It is based on a conversion of L-arginine residue (L-Arg) to L-citrulline residue (L-Cit). The reaction is catalyzed by peptidylarginine deiminases (PAD). The change of L-Arg imino moiety results in a loss of a positive charge. This slight modification can contribute to significant changes in physicochemical properties of proteins, which may also cause a change of their functions. Citrullination is the modification observed in physiological processes such as epidermal keratinization, regulation of gene expression and the reorganization of myelin sheaths. The changes in the efficacy of citrullination may contribute to the pathogenesis of many different diseases including: psoriasis, multiple sclerosis, rheumatoid arthritis and cancer.

KEY WORDS: deimination, peptidylarginine deiminase, citrulline, post-translational modification

List of abbreviations: CARM1 - coactivator-associated arginine methyltransferase 1, **L-Arg-** L-Arginine, **L-Cit -** L-Citrulline, **MAGEA12 -** melanoma-associated antigen 12, **MBP -** myelin basic protein, **p21 -** CDK-interacting protein 1, **PAD -** peptidylarginine deiminase, **PRMT1 - N-**methyltransferase 1, **PRMT5 -** N-methyltransferase 5, **PTN -** pleiothropin, **RA -** rheumatoid arthritis, **TFF1 -** estrogen-responsive trefoil factor 1, **THH -** trichohalin

Citrulline and the reaction of citrullination

L-citrulline (L-Cit) is a ornithine derivative which is related to arginine (Fig. 1). It is found in nature in two forms: as a free amino acid or as an amino acid constituent of proteins.

Three enzymes are involved in the metabolism of free form of citrulline. Two of them originate from the urea cycle: ornithine carbamoyltransferase, which produces L-Cit, and argininosuccinate synthetase which converts L-Cit into argininosuccinate. The third enzyme, NO synthase, produces L-Cit as a by-product in NO production (Curis *et al*. 2005). Due to the importance of the L-Cit in the protein structure, its formation and function will be consider in details. Cit is not encoded by a t-RNA. The only way in which L-Cit can be introduced into proteins is the posttranslational modification of L-Arg - called citrullination. During the reaction, the easily protonated guanidine group of L-Arg is modified into an uncharged carbonyl group (Vossenaar *et al*. 2003). The most important results of this modification are: an altered isoelectric point of the protein, an alteration in the charge distribution and hydrogen or ionic bonds formation in the protein structure. The extensive citrullination of a protein may alter its tertiary structure, interaction with other molecules, cleavage regions, and its

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solubility. The advanced degree of citrullination leads to denaturation of the protein (Tarcsa *et al*. 1996a; Vossenaar *et al*. 2003; Chang *et al*. 2005; Nakayama-Hamada *et al*. 2008).

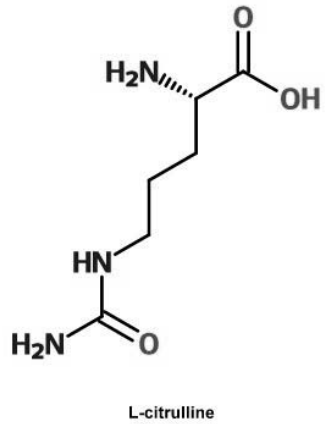

Figure 1. L-citrulline.

Mechanism of reaction

Citrullination is catalyzed by the action of Ca^{2+}-dependent enzymes belonging to the peptidylarginine deiminase family (EC 3.5.3.15). To date, five mammalian PAD genes have been identified. They are localized within the 334,7kb region in cluster 1p36.1. Because of high nucleotide sequence homology (59-71% identity) (Chavanas *et al*. 2006), and the conservative cluster organization it is postulated that PAD are the result of genetic duplications occurring before the divergence of mammalian species. The localization of enzymes and their mRNA with corresponding tissues is shown in Table 1 (Vossenaar *et al*. 2003; Suzuki *et al*. 2007).

As mentioned above, the target of the enzyme is the guanidine group of L-Arg (Fig. 2). Together with the catalytically important cysteine in PAD, it forms an intermediate tetrahedral adduct. Following a nucleophile attack of water molecules, the ammonia molecule is detached. Finally the ketone group is formed (Arita *et al*. 2004). Mammalian PADs have only the ability to convert proteinous L-Arg (or L-methyl-Arg) to L-Cit and free L-Arg is not modified by them (Takahara *et al*. 1986). This possibility possesses only peptidylarginine deiminase from *Porpyromonas gingivalis*, but the enzyme is not evolutionary associated with mammalian isoforms and its catalysis is independent of calcium ions (Rodríguez *et al*. 2010).

Conditions of reaction

The mammalian PAD action is dependent on calcium ions. Under physiological conditions, the Ca^{2+} concentration in the cell is 0,0001 mM, and it is too low to activate the enzymes. For example, PAD2 needs about 100-time higher ion concentration and half of its activity is obtained with 40 – 60 mM Ca^{2+} (Takahara *et al*. 1986; Vossenaar 2004). Because of this limitation, there are several situations in which activation of PAD is possible.

The first possibility concerns extreme conditions such as apoptosis (Asaga *et al*. 1998) or epidermis keratinization (Ying *et al*. 2012). The cell disintegration allows either the calcium ions influx, or enzyme exflux (Vossenaar 2004) to the intercellular space where the ion concentration is appropriate for activation. Alternatively, reservoirs of Ca^{2+} can be released from the mitochondria and endoplasmic reticulum, as is observed in prion disease (O'Donovan *et al*. 2001; Ferreiro *et al*. 2006; Jang *et al*. 2008).

Peptidylarginine deiminase reveals some preferences on the primary and secondary structure of the substrate. For example: Arg located close to Pro is never citrullinated and Arg situated in alpha helix is hardly deiminated. However, N-Arg-Asp-C is the one of most susceptible region for modification, like beta turns (György *et al*. 2006).

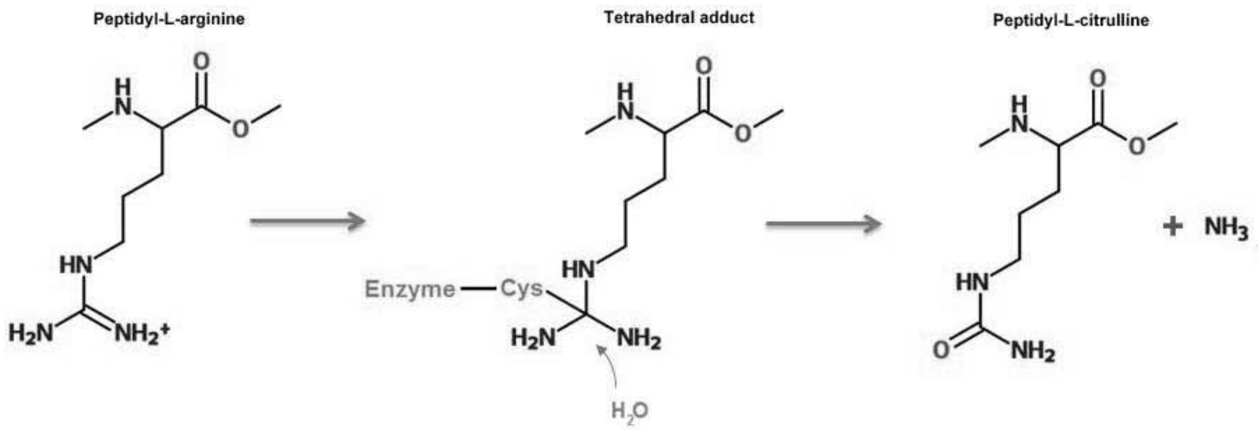

Figure 2. Mechanism of citrullination.

Citrullination in physiology and disease

Citrullination is a process which can occur in physiological or pathological conditions. In physiological processes, deimination play a regulatory role of processes such as the epidermal keratinization (Senshu *et al*. 1996), regulation of gene expression (Karlić *et al*. 2010) and myelin reorganization (Moscarello *et al*. 2002). In the pathogenesis of many diseases the reduction of appropriate level of protein citrullination was observed (psoriasis). On the other hand, the increased levels of L-Arg citrullination in physiological substrates (multiple sclerosis, progression of cancer) and modification of proteins that are not a physiological substrates for PAD was also presented (rheumatoid arthritis)(Vossenaar *et al*. 2003; Chang *et al*. 2011; Takizawa *et al*. 2006).

Epidermal keratinization

The skin is mechanical barrier providing protection against pathogens, skin-penetrating substances, and uncontrolled water loss. A lot of evidences point out the significant role of the protein citrullination in maintaining of the homeostasis and regulation of the keratinization process of the epidermis (Ying *et al*. 2012).

Keratinocytes migrate from the basal to the outer part of the epithelium, there they gradually die forming the stratum corneum. During cell differentiation, the calcium ions influx allows the activation of the three deiminase isoforms: PAD1, PAD2 and PAD3 (Vossenaar *et al*. 2003). Their main substrates include: keratin K1, keratin K10, and filaggrin (Nachat *et al*. 2005; Senshu *et al*. 1996).

Keratin K1 and K10 are fibrillar proteins expressed in the spinous and granular layers (Staquet *et al*. 1987). A deamination within keratin K1 concerns two preferred Arg residues located at Gly-rich subdomains V1 and V2. Within them are located the association sites for loricrin and desmosomes proteins such as desmoplakin (Senshu *et al*. 1999; Steinert *et al*. 1991). The change in the isoelectric point of cytokeratin as the results of citrullination improves its ability to interact with loricrin (Ishida-Yamamoto *et al*. 2000). It is worth mentioning here that due to a lack of Arg, loricrin is not subjected to modification.

Table 1. Expression of peptidylarginine deiminases in human tissues.

Enzymes		Expression sites	
	mRNA length (nts)	mRNA	proteins
PAD1	3846	colon, brain,ES cel, eye, inner ear, placenta, kidney, muscle, thymus, skin	uterus, epidermis
PAD2	4343	Brain, bone marrow, breast, colon, lung, muscle, skin, ovary, synovial membrane, synovial fluid	brain, salivary grand, uterus, macrophage, spleen, bone marrow, skin, synovial fluid, synovial membranę
PAD3	3183	skin, muscle, thymus	hair follicle
PAD4	2263	Brain, eye,fetal liver, bone marrow,kidney, spleen, leukocyte, synovial membranę, synovial fluid	Bone marrow, synovial membrane, synovial fluid, eosinophils, granulocyte
PAD6	2502	Embro, ovary (egg),thymus	Egg, ovary, early embro

The loss of basic character by K1 also affects the interaction with filaggrin - an important protein in the maintenance of epidermal homeostasis. During transition of keratinocyte to corneocyte, calpain 1 releases filaggrin monomers. These monomers are able to interact with keratin. This association results in the formation of an intracorneocyte fibrous matrix (Pearton *et al*. 2002). Some data suggest that citrullination of the filaggrin is crucial to its ability to dissociation and production of Natural Moisturizing Factor (amino acids constitute 52% of the composition)(Tarcsa *et al*. 1996b; Harding & Scott 1983; Ishida-Yamamoto *et al*. 2000).

Cytokeratin K1 and K10 are also linked to trichohalin (THH). It is an essential structural protein responsible for the mechanical strength of the hair follicle inner root sheath (Tarcsa *et al*. 1997). After synthesis THH form insoluble structures which are stabilized by ionic interactions between the alpha helices (Lee *et al*. 1993). During citrullination by PAD3, THH aggregates loosen their structure, making them more susceptible to crosslinking catalyzed by transglutaminase. THH complexed with both keratins leads to the formation of structures that are stable and insoluble in water (Tarcsa *et al*. 1997).

Disturbances in the citrullination are observed in skin diseases such as psoriasis. The disease is characterized by excessive activity of skin cell divisions and defective cornification. Pathomechanism of psoriasis is not fully understood. It is known that cytokeratin K1 has reduced number of citrullinated L-Arg (Ishida-Yamamoto *et al*. 2000).

Regulation of gene expression

Regulation of gene expression takes place on many levels. One of them is the control of transcription via modification of histones (Karlić *et al*. 2010). Histones are basic proteins which provide a framework for organizing the genetic material in a higher-order structure. One of the possible post-translational modification acting as a regulatory process is citrullination. PAD4 is able to modify N-terminal part of the histone H2A, H3, H4 (Hagiwara *et al*. 2005; Mastronardi *et al*. 2006). PAD2 can only modify histone H3 (Cherrington *et al*. 2010).

PAD competes for the L-Arg with methyltransferase, which catalyzes the L-Arg methylation. Addition of methyl groups results in sequential formation of mono-, and then dimethylderivative of an L-Arg. An asymmetric dimethylarginine (both methyl groups on one terminal nitrogen) is created by coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine N-methyltransferase 1 (PRMT1). This modification leads to the activation of gene expression. However, symmetrical dimethylation which is the result of protein arginine N-methyltransferase 5 (PRMT5) activity, inhibits transcription. PAD4 can citrullinate L-Arg, and its monomethylated form. Deimination will decondense chromatin and prevent the creation of dimethylated L-Arg derivative. It is worth mentioning that dimethyl L-Arg is not a substrate for PAD (Thompson & Fast 2006; György *et al*. 2006).

Citrullination contributes to the change in affinity of the transcription apparatus influencing the gene expression *(Wysocka et al*. 2006). Citrullination of histones by PAD4 correlated for example with repression of estrogen-responsive trefoil factor 1 (TFF1) gene and apoptosis-associated CDK-interacting protein 1 (p21) and OKL38 genes. PAD2 is involved in the regulation of pleiothropin (PTN) and melanoma-associated antigen 12 (MAGEA12) genes (Cherrington *et al*. 2012).

PAD overexpression and changes in their subcellular localization is often accompanied by certain types of cancer (Mohanan *et al*. 2012). Increased levels of PAD4 and its activity is presented in invasive carcinomas like lung adenocarcinomas, esophageal carcinomas with squamous differentiation, colorectal adenocarcinomas and bladder uterine carcinomas etc. (Wang *et al*. 2010). The progression of cancer is also related to the change of PAD2 localization. In the normal breast tissues PAD2 is localized in both the cytoplasm and the nucleus. Changing the location of nuclear PAD2 in certain types of cancer may cause changes in gene expression and cause malignant transformation (Mohanan *et al*. 2012).

Creation and reorganization of myelin sheaths

A myelin sheath is formed by oligodendrocytes in the central nervous system, and Schwann cells in the peripheral nervous system. The main purpose of the myelin sheath is electrically isolation of axon and an increase in the speed of propagation of an electrical impulse along the myelinated fiber (Kursula 2008).

Myelin sheaths are composed of proteins, like myelin basic protein (MBP) and lipid components. The protein molecules are basic. They interact with negatively charged lipids like gangliosides and phosphatidylserine. Lipid-protein interaction is a key element in the sheath formation (Boggs *et al*. 1999). Native MBP are able to form a tight, compact structure. Such a structure is not conducive to its reorganization. The mutual interaction of molecules may be affected by frequent post-translational modifications within the MBP including citrullination, deamidation and methylation. The change in the isoelectric point of the protein after modification significantly influences the sheaths relaxation (Beniac *et al*. 2000).

The enzyme involved in the citrullination of MBP is PAD2. The highest PAD2 expression is observed in the gray matter and the hypothalamus (Kubilus & Baden 1983). It is known that the amount of deiminated MBP changes dramatically during life. In children under 2 years old, almost all MBP are modified. The degree of citrullination correlates with the observed brain plasticity (Moscarello *et al*. 1994). The number of modified proteins decreases with age. In the adult brain, the amount of citrullinated MBP remains constant and represents about 20% of the total pool of MBP (Moscarello *et al*. 2002).

Hyper-citrullination of proteins is observed in various neurodegenerative diseases such as multiple sclerosis. Modification of MBP applies not only to the percentage of modified proteins (increase from 20% to 45%), but also to the amount of citrullinated L-Arg (increase from 6 to 18 residues) (Moscarello *et al*.

1994; Wood *et al*. 1996). It is noted that excessive citrullination may occur during a reduced methylation as a result of a lowered methyltransferase activity (György *et al*. 2006).

Hyper-citrullination of MBP contributes to the development of the autoimmune response. Modified MBP are more susceptible to degradation of cathepsin D. PAD2 is localized in CNS myelin and presents elevated activity in multiple sclerosis (MS) (Berlet 1987). The reaction is approximately 35x faster in comparison to native MBP (Cao *et al*. 1999). Peptide released by cathepsin D contains an immunodominant epitope (Pritzker *et al*. 2000). Immune cells such as lymphocytes infiltrate into nerve tissue cause: oxidative stress, local inflammation and myelin sheath destruction underlying demyelinating disease (Whitaker *et al*. 1980).

Blood clot formation

The clot is a structure formed by the components of the blood to stop bleeding and repair the damaged blood vessel. One of the main proteins involved in the blood coagulation cascade is fibrinogen. Structurally, this is a dimeric glycoprotein. The release of fibrinopeptides A and B from fibrinogen, catalyzed by thrombin, results in the formation of monomers with exposed polymerization sites. Monomers organize themselves spontaneously and form a labile and then cross-linked stable fibrin. Finally, red cells and platelets adhere to resulting structure and form a clot (Blombäck *et al*. 1978; Furie & Furie 1988; Nakayama-Hamada *et al*. 2008).

Extravascular clot formation usually accompanies inflammatory processes for instance rheumatoid arthritis (RA). RA is a systemic autoimmune disease characterized by inflammation of peripheral joint which leads to cartilage destruction and joint dysfunction (Firestein 2003). During the infiltration of inflammatory synovium, monocytes differentiate into macrophages and subsequently become activated. Sustained activation makes them susceptible to programmed death. During the macrophage apoptosis, PAD2 and PAD4 are activated and then leaked into the synovium *(Rodenburg et al.* 2000; Vossenaar 2004). Damaged cell products stimulate the retraction of endothelial cells, which facilitates the extravasation of fibrinogen and other plasma components (Méchin *et al*. 2007). Citrullination can indeed occur within the rheumatoid synovial tissue with many different L-Arg residues citrullinated in different proteins including fibrinogen (Okumura *et al*. 2009; Vossenaar 2004).

Cleavage sites for thrombin are located on the N-terminus of Aα and Bβ chains of fibrinogen. It falls between Arg^{16}-Gly^{17} in the Aα chain and Arg^{14}-Gly^{15} in Bβ chain. Deimination blocks the releasing of fibrinopeptides because of L-Arg modification and thus prevents the polymerization of fibrin. Citrullinated fibrinogen in this case acts as an uncompetitive inhibitor of thrombin reaction (Nakayama-Hamada *et al*. 2008). In addition, certain fibrinogen molecules can be converted to fibrin before deimination. It is perhaps possible due to the increased level of thrombin in the synovial fluid (So *et al*. 2003)

After fibrin citrullination, a reduction ability to degradation it by plasmin can also be observed. This serine proteinase cleaves the peptide bond near basic amino acids such as Lys and Arg. Deimination reduces the number of potential degradation sites (Sebbag *et al*. 2004).

Currently, it is unknown whether the formation of fibrinogen deposits is a primary or secondary cause of the disease. However, it is known that fibrinogen can stimulate an immune response in two ways; directly across immunogenic citrullinated alpha and beta chains (Masson-Bessière *et al*. 2001) and indirectly by stimulating production of IL-1, IL-8, IL-13 and TNF-alpha by macrophages, resulting in extravasation of fibrinogen and a cyclic process (Rubin & Sønderstrup 2004).

Conclusion

Citrullination has been observed in many physiological and pathological processes. Modification can drastically change the properties and thus the function of the protein. However, we should note, that the citrullination have mainly regulatory functions and its role in pathogenic processes is possible only under certain conditions.

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